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Observations on the Phenology and Reproductive Success of the Critically Endangered *Nervilia nipponica* (Orchidaceae) in Kochi Prefecture, Japan

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Nervilia nipponica is critically endangered and poorly known throughout its range in Japan. In order to illuminate details of the species' life history, observations on the phenology and reproductive success of two large populations in Kochi Prefecture were made from 2003-2004. Patterns of emergence were comparable and individuals in both populations exhibited a highly synchronous phenology. The average individual flowering period lasted 12.3 and 11.0 days respectively at the two sites, and dehiscence of fruit capsules occurred 25.6 and 25.3 days after flowering. Only 34.2% and 28.4% of plants at the two sites successfully flowered, but rates of seed set were 38.2% and 52.4% despite the virtual absence of a nectar reward and the lack of pollen vectors, probably indicative of self-pollination. The failure of individuals marked in 2003 to re-emerge, as well as the appearance of unmarked individuals in 2004, suggests that the species has a capacity for dormancy and/or rapid propagation via the tuber. Evidence for the latter is provided by the excavation of 89 tubers from 58 individuals, representing an average rate of increase of 1.5 per individual per year. Recommendations are made for ongoing studies towards the development of a conservation strategy for the species.

Key words: Conservation, *Nervilia nipponica*, phenology, population, reproductive success

Field observations have been highlighted as the primary means of generating a demographic understanding of threatened orchid species so as to determine both conservation status and the life history stages that might be limiting distribution, population growth and persistence (Tremblay & Hutchings 2003). Long-term studies (*i.e.*, several years to decades) have been conducted on a select number of terrestrial species, primarily in Europe and North America, in which individually marked plants or permanent vegetation plots are monitored typically on an annual basis to provide insights into life histories and/or population dynamics (Tamm 1991, Wells & Cox 1991). Studies utilizing more intensive monitoring strategies over shorter time frames (*i.e.*, one to two years) are less frequent in the literature, though they have been used to reveal irregular patterns of emergence of plants of *Ophrys sphegodes* Mill. over a single growing season (Sanger & Waite 1998), and changes in the partitioning of biomass among different plant parts in regenerating individuals of *Dactylorhiza fuschii* (Druce) Vermeul. (Leeson *et al.* 1991). In addition to emphasizing the dynamic nature of terrestrial orchid populations over short time frames, such approaches are likely to be useful in establishing annual phenology and baseline data on reproductive biology, particularly for rare species about which very little is known.

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Nervilia nipponica Makino is an inconspicuous terrestrial orchid that has been reported from a limited number of sites along the Pacific seaboard of Japan, from Tokyo to the Ryukyus, and Taiwan (Masamune & Satomi 1972, Sawa 1979, Hoshi & Katsuyama 1992, Su 2000). In Japan, its occurrence is changeable and sporadic with populations typically being isolated and prone to sudden local extinctions (for example in Kanagawa Prefecture, T. Katsuyama pers. comm. and in Miyazaki Prefecture, T. Minamitani pers. comm.). In addition, most reports for the species refer to population sizes of tens of individuals at most (Suzuki 1955, Ogawa 1969, Hoshi & Katsuyama 1992). The small total number of individuals and extant sites confirmed for the species in Japan have led to *N. nipponica* being rated as Critically Endangered (CR; IUCN 2001) at both the Prefectural (Makino Memorial Foundation of Kochi Prefecture 2000, Red Data Book Committee of Miyazaki Prefecture 2000, Kagoshima Prefecture Department for Environmental Protection 2003) and national level (Environmental Agency of Japan 2000).

Like all members of the genus, *Nervilia nipponica* consists of a subterranean tuber that sequentially produces a single flowering scape and then, at or after seed set, a single cordate leaf within a single growing season, a growth habit known as hysteranthy; all above-ground parts (*i.e.*, the leaf and petiole) die back over the winter. In common with other members of section *Linervia* (Schlechter 1911), the scape bears just one flower and elongates during fructification.

In the most detailed study of the species to date, Sawa (1979) reports that the flowers of most flowering individuals at a site in Kochi Prefecture did not open widely and that pollination was marked by the erect or horizontal flower becoming first pendulous, before resuming an erect position during scape elongation. A small number of cultivated plants were induced to undergo this sequence through either artificial self-pollination or through manually shaking the plant (and thereby possibly dislodging the pollinia onto the stigma), providing anecdotal evidence for the role of self-pollination for seed set *in situ* (Sawa 1979). The population observed in that study flowered in May, with leaves being produced following capsule dehiscence in June. Elsewhere, observations of a small population on Oshima, Tokyo, were seen to flower in mid-May and to have produced leaves by mid-June (Ogawa 1969); the timing of the same phenological events was similar for a population in Kanagawa Prefecture (Hoshi & Katsuyama 1992).

Though these studies provide a platform for further research, none involved regular observations of individually marked plants to gauge the relative abundance of individuals passing through successive phenological stages, the length of key phenological events, or the fate of individuals within and between growing seasons. A demographic understanding of the species' phenology and reproductive biology might facilitate in the short term intervenient measures such as establishment of *ex situ* collections, supplementary hand pollinations, seed collection or (for particularly vulnerable populations) relocations, as well as promote more targeted research (*e.g.,* population genetics, *in vitro* propagation, *etc.*) towards an integrated recovery programme in the long term. Moreover, whereas most long-term studies of other terrestrial orchids have relied on a single annual census, matters are complicated for *Nervilia nipponica* by its hysteranthous growth habit, such that reproductive and photosynthetic populations cannot be censured on the same site visit. It therefore remains necessary to establish the best strategy for long-term monitoring of the species.

The objectives of the present study were therefore: to (i) provide detailed information on the phenology and background fluctuation in numbers of *Nervilia nipponica* throughout a single growing season; and to (ii) provide baseline data on modes of reproduction including flowering and fruit rate, seed production and capacity for vegetative propagation via synthesis of new tubers, a prominent feature of the reproductive biology of African members of the genus (Pettersson 1991). The data is intended as preliminary to long-term population studies and is discussed in terms of its relevance to subsequent conservation efforts for the species.

Materials and Methods

Study sites

Of the handful of sites known in Kochi Prefecture, two populations of *Nervilia nipponica* were selected for study because of their accessibility and large size, features which facilitated regular field visits and permitted the collection of unusually large data sets for the species. The two populations occur on the same southeast-facing slope of a low mountain (212 m altitude at summit) situated 5 km from the Pacific coast of Kochi City. Population KL, the larger of the two populations, occurs between c. 40-50 m a.s.l. and covers an area of approximately 35 m × 40 m. The site here consists of secondary woodland composed of a mixture of broadleaved evergreen (*Quercus glauca* Thunb. ex Murray, *Ligustrum japonicum* Thunb., *Camellia japonica* L. and *Lithocarpus glabra* (Thunb. ex Murray) Nakai) and deciduous (*Prunus jamasakura* Sieb. ex Koidz. and *Rhus succedana* L.) trees to 20 m ht, many of which have resprouted from stools cut back about 50 years ago. The second population, population KU, is situated between c.75-80 m a.s.l. and covers an area of approximately 28 m × 24 m. The site at KU consists of an abandoned softwood plantation of *Cryptomeria japonica* (L. fil.) D. Don and *Chamaecyparis obtusa* (Sieb et Zucc.) Endl., which has attained about 17 m ht. Other woody species including *Ligustrum japonicum* Thunb. and *Cinnamomum japonicum* Sieb. ex Nakai have seeded in.

Despite differences in species composition, the habitats at the two sites are similar in the following respects: (i) the presence of a thick leaf litter and soils with a high red clay content (apparent from a depth of c. 6 cm), which together generate high, stable moisture levels in the upper soil horizons in which tubers of *Nervilia nipponica* are found; and (ii) a forest floor cast in shade by a mature, predominantly evergreen canopy but exposed to a regime of temporally and spatially complex sunflecks due to the absence of a shrub layer or undergrowth directly above *N. nipponica* patches.

Marking and observations

All plants at population KL (n = 524) were marked on 25-27 September 2003 by inserting a numbered stainless steel tag into the ground within 3 cm of each leaf. Similarly, all plants at population KU (n = 170) were labeled on 11-12 October 2003. Irregular site visits thereafter in 2003 confirmed that both populations were in decline with all above ground parts having undergone complete senescence by 6 December 2003. The timing of the appearance of new buds in spring 2004 was preempted by making daily observations of a small number of *Nervilia nipponica* plants cultivated in the Makino Botanical Garden. New growth in these plants was first observed on 9 April 2004, somewhat earlier than plants *in situ* at population KL and population KU. In order to avoid trampling in densely populated areas and to allow some plants to be used in other associated studies (of floral biology, seed set *etc.*), not all plants were included in the phenological study: 416 plants were monitored at population KL, and 86 plants were monitored at population KU.

Regular (± weekly) field observations of marked plants commenced on 15 April 2004 and continued throughout one complete growing season until no plant remained above ground. On each site visit, each plant was observed in turn and assigned

TABLE 1. Phenological stages and descriptions used to score plants in the study.

Stage	Phase	Description
1	Scape	Scape bud visible above ground, to c.20mm tall.
2	Scape	Flower bud visible beyond (and free of) upper cataphyll. 20–50mm tall.
3	Flower	Flower erect, pedicel visible, sepals parting slightly to reveal white petals. >30mm tall.
4	Flower	Bract visible, flower ± horizontal, sepals open, petals not at all, very weakly (to reveal lip) or weakly spreading (to reveal column). 45–110mm tall.
5	Flower	Flower drooping at pedicel, sepals and petals very weakly spreading or closed. 45–110mm tall.
6	Fruit	Flower parts closed, ovary slightly swollen and scape internodes (particularly uppermost) and pedicel extending. 50–160mm tall.
7	Fruit	Ovary swollen and held ±horizontal or vertically upwards by pedicel. Yellowish fissure lines along length of capsule. 80–200mm tall.
8	Fruit	Capsule burst releasing many dust-like seeds. 80–200mm tall.
9	Immature leaf	Tapering light green leaf bud visible above ground, to c. 25mm tall.
10	Immature leaf	Leaf involute or only slightly unfurled, blade and petiole indistinct, 20–45mm tall.
11	Immature leaf	Petiole and leaf blade distinct, the latter incompletely unfurled, margin sinuate, 20–60mm tall.
12	Mature leaf	Leaf blade fully open, usually horizontal, green to deep green, 5 (rarely 7) primary veins. 15–55mm wide, 15–50mm long. Petiole erect, to 80mm long.
13	Senescence	More than 1/3 of the leaf area yellow, secondary reticulate veins visible, petiole wilting.
14	Senescence	Leaf entirely yellow and no longer photosynthetic, petiole collapsed.

to one of a sequence of discrete stages according to its phenological state. Each stage was delineated through ongoing field observation, complemented by more frequent monitoring of plants in cultivation at the Makino Botanical Garden. Notes were also made on plants affected by herbivory or other stress, as well as those that withered prior to completing a particular phenological stage. Whenever possible, observations at both sites were made on the same day, though due to practical limitations some site visits (11 of a total of 30 per population) were carried out over two consecutive days.

Notes on the presence/absence of insect visitors were made in the course of site visits during the flowering season. The production of nectar by flowers of *Nervilia nipponica* was checked by dissecting 5 flowers under a binocular microscope. The number of seeds contained in a single fruit was estimated by dissecting 5 ripe pods under low power magnification and counting the contents against a squared paper background.

During the monitoring period, leaves which had not been marked in 2003 appeared above ground at both population KL and population KU. Though these plants were not observed regularly as part of the phenological study, they were marked and counted in August 2004 in an assessment of demographic change from 2003-2004 at the two study sites.

To gain a better understanding of the development of below-ground parts over one growing season, 4 plants not involved in the phenological study were excavated from the soil every fourth site visit (17 June, 15 July, 12 August, 21 September and 2 November) at KL. In addition, unavoidable construction work at KL facilitated the excavation of individual plants at two microsites (constituting two patches of *Nervilia nipponica*) on 15-21 November 2004. The number of tubers recovered per marked individual was recorded at both microsites in an assessment of the species' potential for clonal reproduction.

Results

A total of 14 stages were recognised to score plants on each site visit (Table 1; Figs. 1, 2 & 3). These describe 6 successive phenological phases (scape, flower, fruit, immature leaf, mature leaf and senescence) covering the complete above-ground phe-

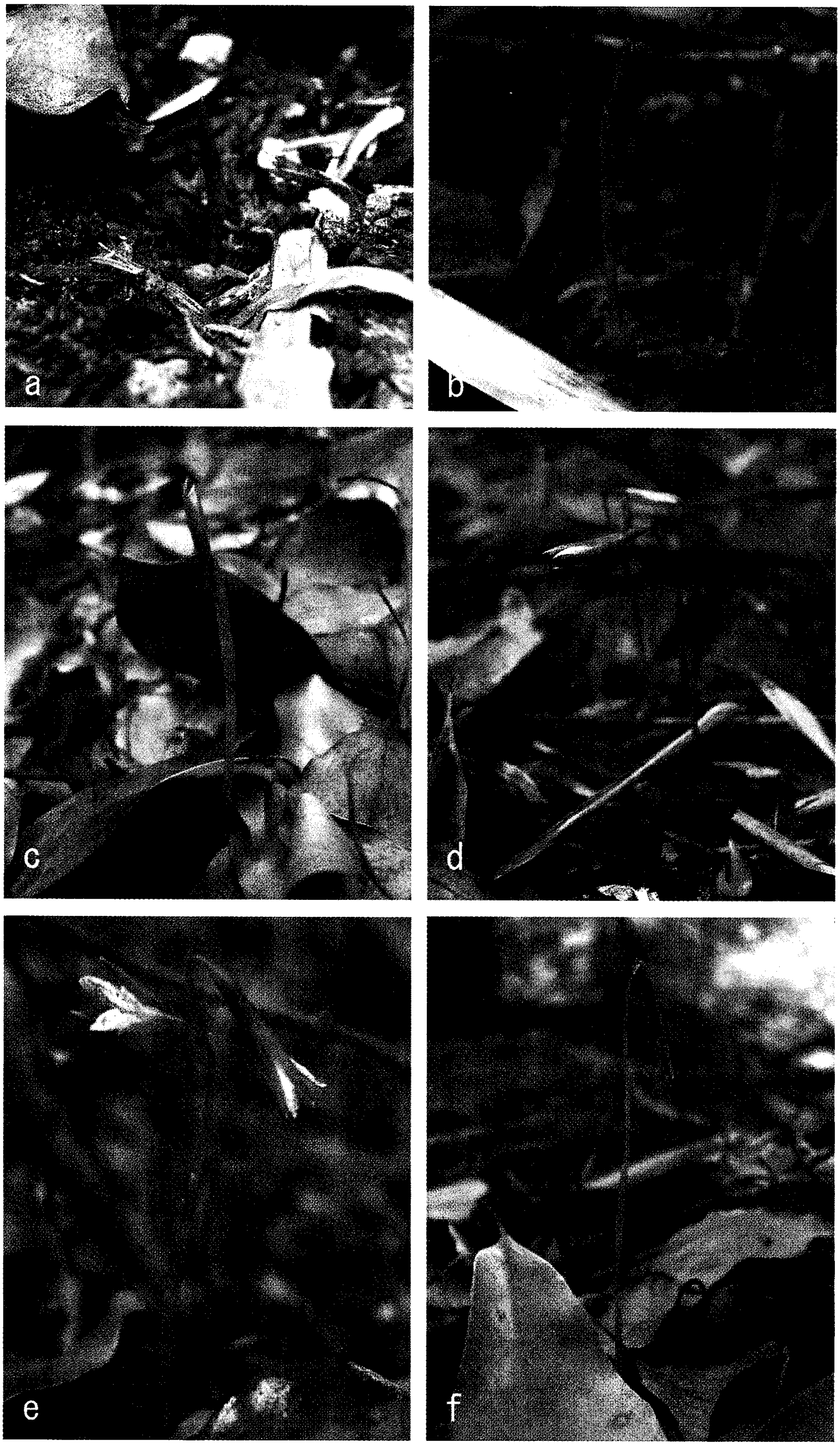

FIG. 1. Phenology of *Nervilia nipponica*: scape and flower. (a) Stage 1. (b) Stage 2. (c) Stage 3. (d) & (e) Stage 4. (f) Stage 5.

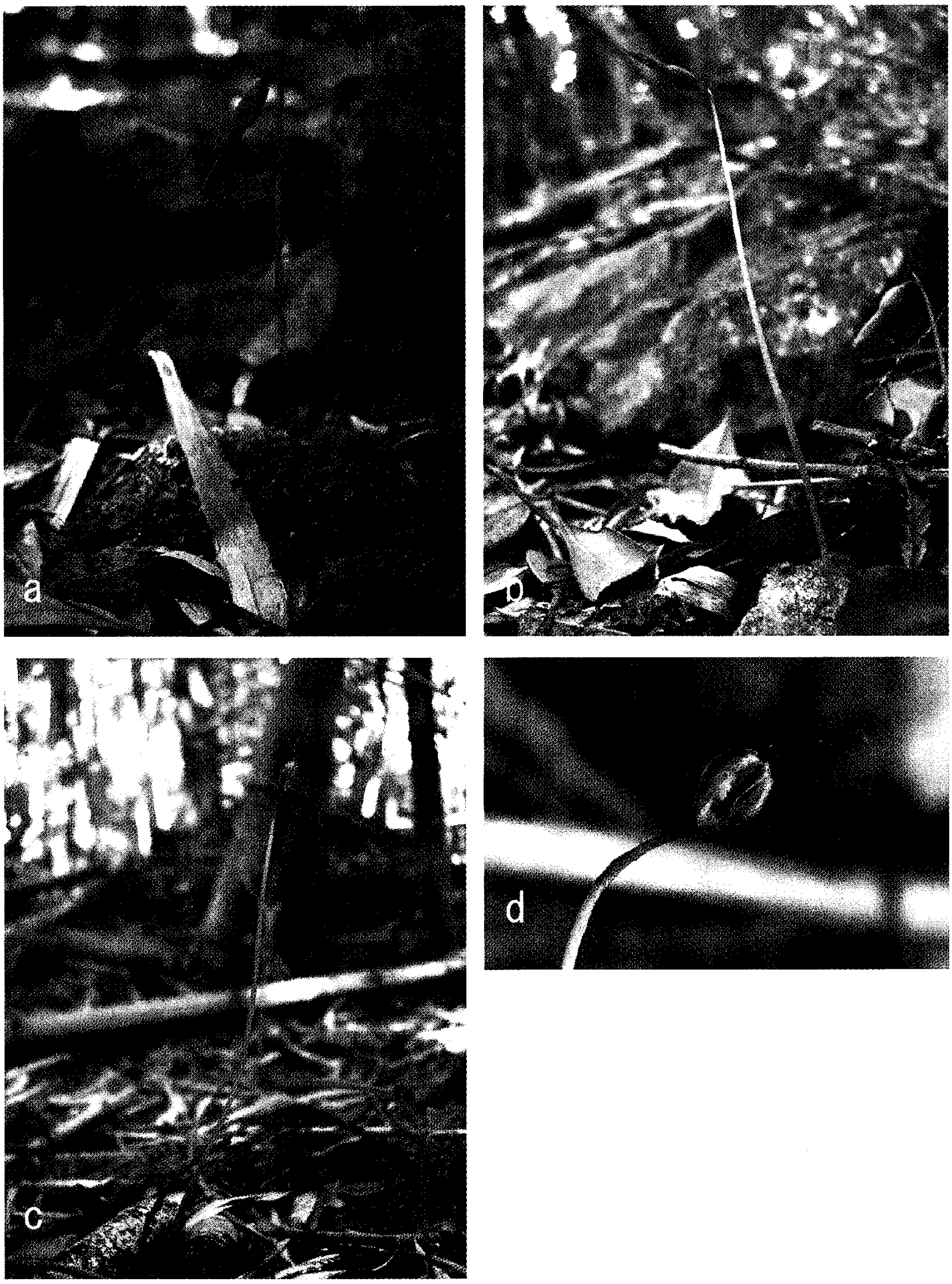

FIG. 2. Phenology of *Nervilia nipponica*: fruit. (a) Stage 6. (b) Stage 7. (c) & (e) Stage 8.

nology of *Nervilia nipponica*.

Phenology

Fig. 4 shows the composite number of individuals falling into each of these phases at population KL and population KU throughout the monitoring period. Buds of new flowering scapes (stage 1) were first observed at both sites on 22 April. Flowering (stages 3-5) commenced two weeks later on 6 May and continued until 1 June, a period of 27 days. The size of the flowering population peaked on 15 May at population KL (n = 79) and on 16 May at population KU (n = 16). Fructification (stages 6-8) commenced on 25 May at both sites, and all fruiting individuals had gone to seed by 17 June at population KL and by 25 June at population KU. Thus fruit

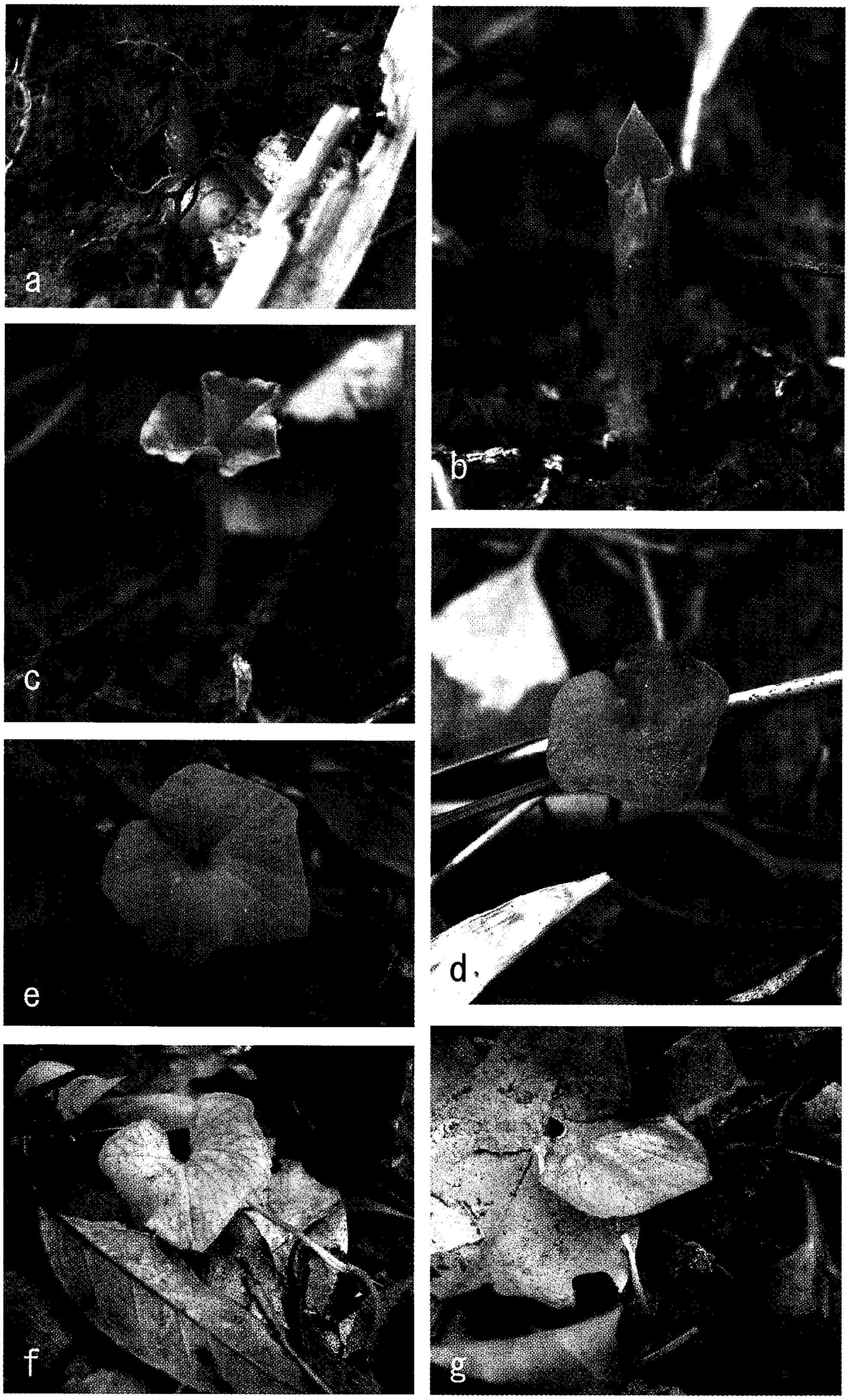

FIG. 3. Phenology of *Nervilia nipponica*: leaf and senescence. (a) Stage 9. (b) Stage 10. (c) Stage 11. (d) & (e) Stage 12. (f) Stage 13. (g) Stage 14.

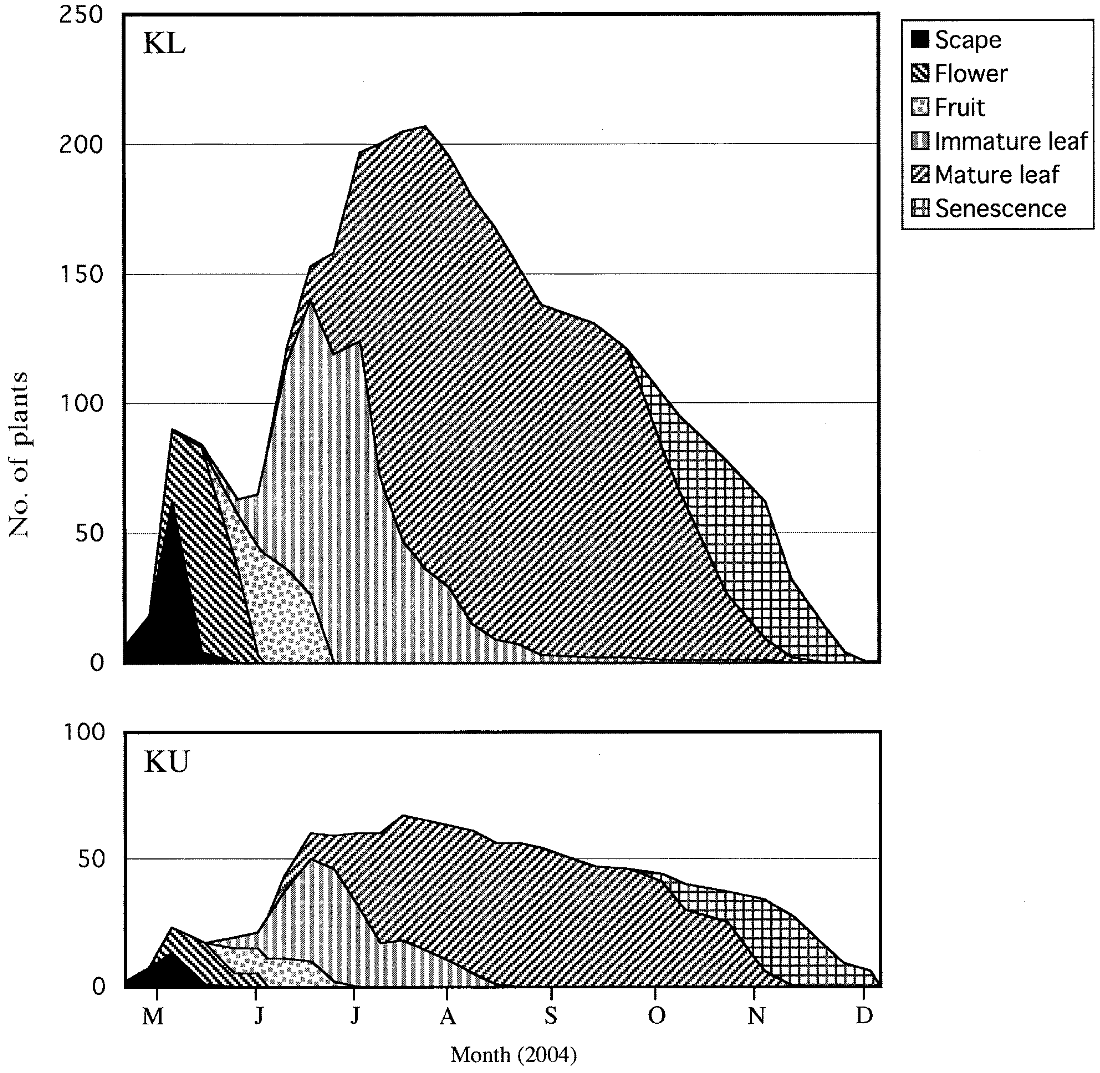

FIG. 4. Above-ground phenology of two populations of *Nervilia nipponica* in Kochi Prefecture, Japan.

maturation and dehiscence occurred over a period of 24 days at population KL and 32 days at population KU. New leaf buds (stage 9) were first observed on 6 May at population KL and on 25 May at population KU, and most plants at both sites had developed into mature leaves (stage 12) by 8 July. Leafing populations peaked on 22 July at both population KL (n = 207) and population KU (n = 65); both populations were in decline after this date. Senescence was apparent at both population KL and population KU from 2 October, and all leaves had died and disappeared at population KL by 3 December and by 6 December at population KU. Since all plants were individually labeled, the repeat observations also permitted calculation of the average length of time a single plant spends in any one key phenological phase (Table 2).

The numbers of marked plants that re-emerged in 2003 at either site as flowers, leaves or both are given in Table 3. In addition to the plants marked in 2003, a total of 353 and 211 ‘new’ plants appeared respectively at population KL and population KU in 2004.

Reproductive success

Of the total marked population observed above ground at population KL and population KU, only 89 (34.2%) and 21 (28.4%) plants respectively actu-

TABLE 2. Average lengths of key phenological phases at the two study sites. Only those plants that were seen to continue onto subsequent stage were included in the calculation (numbers of plants used to calculate average given in parentheses).

	Length in days ±SD (n)	
	KL	*KU*
Scape (stage 1 – 2)	8.6 ±1.9 (47)	9.2 ±3.4 (11)
Flowering (stage 3 – 5)	12.3 ±3.9 (39)	11.0 ±1.6 (13)
Fructification (stage 6 – 8)	16.9 ±3.7 (34)	18.5 ±4.5 (11)
End of flowering to dehiscence (stage 5 – 8)	25.6 ±6.3 (26)	25.3 ±5.0 (11)
Immature leaf (stage 9 – 11)	23.3 ±10.6 (67)	23.0 ±9.1 (28)
Mature leaf (stage 12)	90.4 ±21.6 (78)	96.3 ±23.4 (33)

ally flowered (stages 3 to 5). The maximum number of plants observed in full anthesis (stage 4) on any one site visit was 44 at population KL and 6 at population KU, constituting flowering synchrony rates (for the whole population) of 16.9% and 8.1% respectively. During the course of the present study no insect pollinators were observed on flowers of *Nervilia nipponica* at either site, and only minute amounts of nectar were found in dissected flowers (n = 5) harvested from plants not involved in the phenological study (data not shown). Thirty-four plants at population KL and 11 plants at population KU produced seed-bearing capsules which dehisced, representing fruiting rates of 38.2% and 52.4% respectively. Five ripe but non-dehisced capsules were found to contain approximately 1540, 1780, 1950, 2100, and 2180 seeds respectively. These figures are underestimates because some of the dust seeds were unavoidably lost when opening and moving the capsules.

Segmented runners which extended ± horizontally from the subterranean stem above the tuber began to develop on all leafing individuals examined (n = 4) in mid-June. Between 2-4 runners were observed per plant excavated in mid-July (Fig. 5(a)).

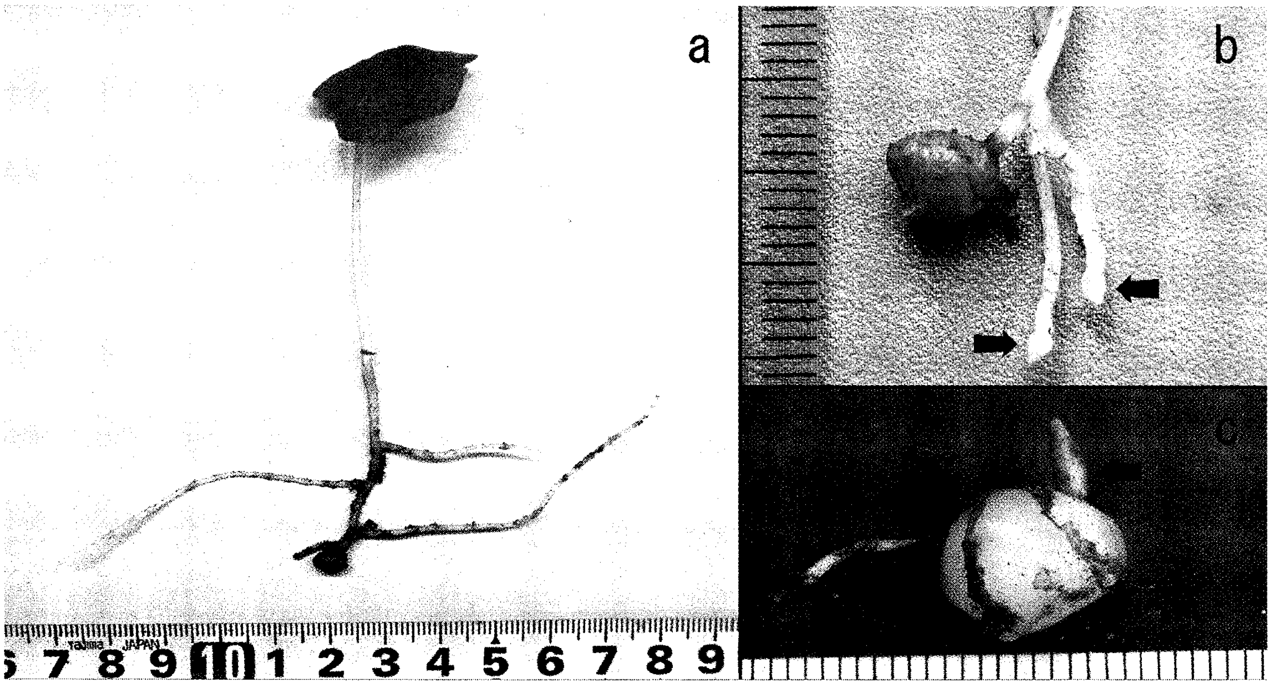

FIG. 5. Subterranean parts in *Nervilia nipponica*. (a) Four runners extending laterally from the subterranean stem (15 July 2004). (b) Terminal internodes of runners enlarging to form daughter tubers (arrows; 12 Aug. 2004). (c) Daughter tuber with bud (arrow) at apex opposite attachment to severed runner (21 Nov. 2004). Fine increments in (a), (b) and (c) = 1 mm.

TABLE 3. Number of plants marked in 2003 that failed to re-emerge, emerged as flowers, leaves, or both during the study period in 2004. Numbers in parentheses denote percentages of the total monitored population (totals = 416 plants at population KL, 86 plants at population KU).

Population KL

		Leaf	
		−	+
Flower	−	156 (37.5%)	162 (38.9%)
	+	37 (8.9%)	61 (14.7%)

Population KU

		Leaf	
		−	+
Flower	−	12 (14%)	45 (52.3%)
	+	5 (5.8%)	24 (27.9%)

By mid-August, the runners had attained lengths of 2-15 cm, and daughter tubers had begun to form at their tips through radial enlargement of the apical-most 1-3 internodes with reduced lateral extension (Fig. 5(b)). The new tubers continued to enlarge in September, and had attained diameters of <2-5.5 mm by early November. A single conical bud had developed at the apex (*i.e.*, opposite the attachment to the runner) on some tubers by late November (Fig. 5(c)). It is inferred that the stem develops from this bud the following year. As plants began to senesce from October, the runners also degraded, leaving the new tubers as independent units. These observations suggest that a single plant might produce up to 4 new tubers over a single growing season.

In mid-November, 32 and 26 plants of *Nervilia nipponica* were excavated respectively at the two microsites at population KL. As most plants had already senesced by this time, petioles and runners were mostly absent and individual tubers were found in the F, H and A_1 strata of the soil. At the first of the two microsites, 49 tubers were recovered, representing an average rate of increase of 1.5 per individual. At the second microsite, 40 tubers were recovered, also representing a rate of increase of 1.5 per individual. These observed rates of clonal regeneration fall short of those predicted from the above observations of runner production.

Herbivory and stress

The numbers of plants eliminated from either population due to herbivory or other stress at successive phenological stages are given in Fig. 6. At population KL, 49 plants that produced scape buds (of a total of 98, 50%) were prevented from potentially setting seed due to herbivory. A further 15 withered. Similarly, 18 of the 29 plants (62.1%) that produced scape buds at population KU were lost through herbivory prior to seed set. All plants not affected by herbivory at population KU successfully set seed, *i.e.*, none withered prior to dehiscence. Larvae of the moth *Ercheia niveostrigata* (Lepidoptera: Noctuidae) were found feeding on the scapes of 11 plants at population KL and population KU.

Only 82 of the 223 plants at population KL and 34 plants of the 69 at population KU that produced leaf buds (stage 9) were seen to complete the growing season through senescence (stages 13 and 14). One hundred and sixteen leaves (52.0%) at population KL and 26 (37.7%) at population KU were prevented from doing so through herbivory. The remainder were eliminated through stress either in long dry spells (notably in July-August), or by one of several severe typhoons (in September-October). The main herbivores on leaves were unidentified moth instars.

Discussion

Plants at both sites underwent a predictable sequence of phenological development with the two populations showing a highly comparable trend in the

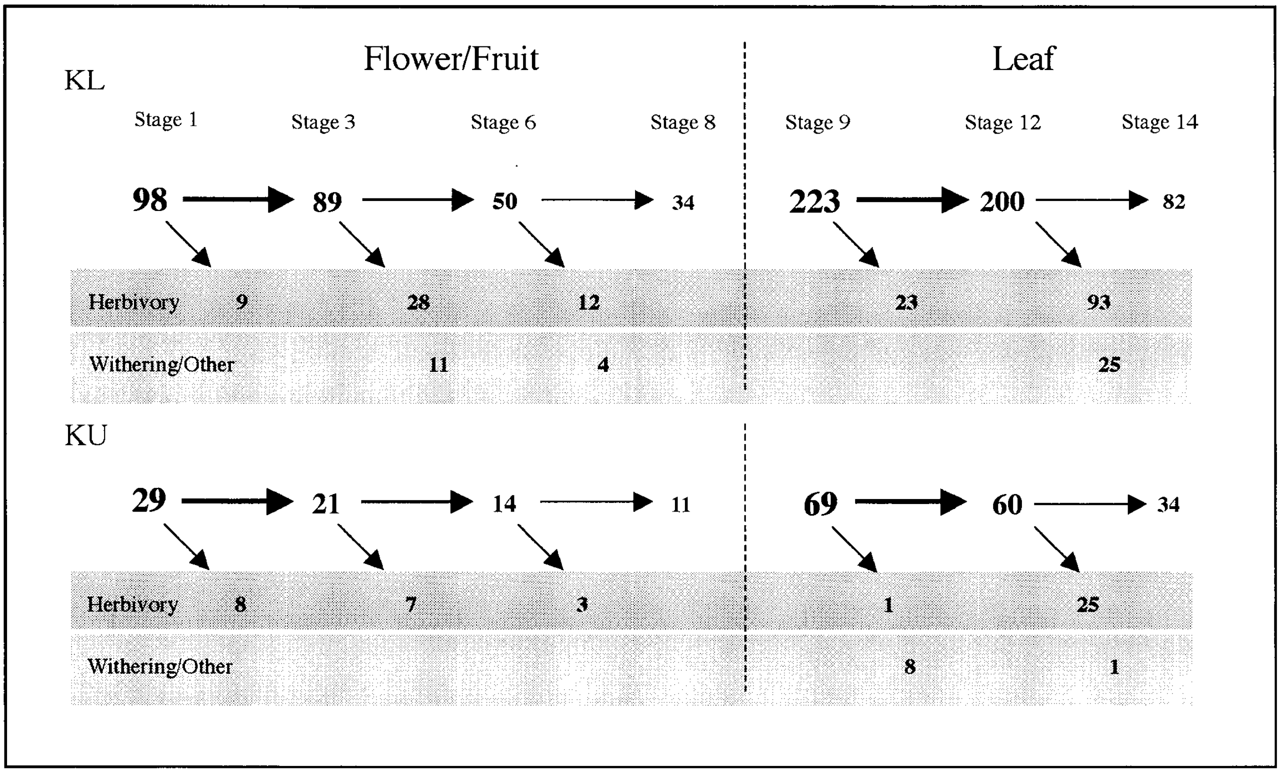

FIG. 6. Numbers of individuals at successive stages and of those lost due to herbivory and withering.

timing and length of each phase and the proportion of individuals involved. Flowering, fruiting and leaf flush occurred at times similar to those given in other accounts for the species in other parts of its range (Ogawa 1969, Sawa 1979, Hoshi & Katsuyama 1992), and thus this pattern of emergence is very likely descriptive of the species as a whole. Unlike *Ophrys sphegodes*, a single population of which was found to consist of up to three distinct cohorts of emerging plants (Sanger & Waite 1998), all individuals of *Nervilia nipponica* observed in the present study exhibited a highly synchronized pattern of emergence. However, not all plants were found to be truly hysteranthous over a single growing season, with only 61 plants (23.5% of emerging plants) at population KL and 24 plants (32.4%) at population KU producing both a flower and a leaf (Table 3). Additional studies are required to determine the reason(s) for the failure of plants to enter both flowering and leafing phases, though the production of a bud on the tuber by the end of the preceding season and nutrient status over the winter could be decisive factors.

For further studies assessing long-term population status and other population-level parameters such as half-life, flowering frequency and tuber dormancy, it is recommended that monitoring and marking of new recruits is conducted at least twice annually, in mid-May and late-July when the population is at the peak of its reproductive and photosynthetic phases respectively. Some plants may have already been lost from the above-ground phase through herbivory or withering by the time of these census dates, however, potentially leading to underestimates of total population size and misinterpretations of individual plant life histories.

Only 260 and 74 of the plants marked in 2003 re-emerged respectively at population KL and population KU in 2004, representing potential declines of 37.5% and 14% over that period. However, 353 and 211 unmarked 'new' plants appeared above ground at the two sites in 2004. Though some may have been present the previous year but had already disappeared by the time the populations were marked in 2003, the large numbers involved imply that a significant proportion of wild populations undergo high turn-over in the short term. Such fluctuation in the status of individual plants could be caused by rapid recruitment through either seed or tuber, or by plants entering or emerging from a dormant subterranean phase, as well as through death.

The observed average rate of annual increase of 1.5 per individual provides some evidence for the capability of populations to ensure replacement and growth via clonal reproduction. Since the small (2-5mm diameter) and inconspicuously coloured tubers were very difficult to locate in the soil, true rates of tuber production are likely to be higher. Conversely, rates of increase may not be as high as those expected from observations of runner production throughout the growing season since some new tubers may not survive. In addition, though parent tubers could be identified on some plants excavated in November because a leaf petiole remained, it was not always possible to tell whether the parent tuber survived for a subsequent year's growth, or whether they had already degraded. However, given the patchy nature of the populations and the observation that all plants produce new tubers, vegetative propagation might be viewed as the primary means of reproduction in the short term and at the microsite level. Further work is required to determine the survivorship of tubers from one season to the next, their capacity for dormancy, as well as their average lifespan. The use of genetic markers are advocated to determine the level of clonality at the population level and in turn define the scale at which conservation efforts should focus.

In addition to earlier observations (Sawa 1979) that flowers of *Nervilia nipponica* spread only weakly and that plants are capable of seed set *ex situ* in the absence of pollinators, plants in the present study were also found to be effectively non-rewarding and failed to attract any insect visitors. Opportunities for panmixis were also limited by the small number of flowers synchronously at full anthesis. Natural fruit conversion rates were nonetheless high: compared with other non-rewarding terrestrial orchid species, fruit set in *N. nipponica* was well above the average values calculated for all geographic regions excluding the temperate southern hemisphere (Neiland & Wilcock 1998). Furthermore, if the 40 plants at KL and 10 plants at KU destroyed by herbivory after flowering and prior to capsule dehiscence (*i.e.*, at any of stages 3-7) are excluded, hypothetical fruiting rates as high as 69.4% and 100% are generated respectively for the two sites. Withering of scapes prior to fructification, an outcome Sawa (1979) attributes to the absence of pollination, also stunts fruiting rates but is relatively uncommon. These figures are very likely indicative of autogamy and/or agamospermy, and sit well with the paradigm that non-rewarding orchids are able to positively modulate fruiting success through pollinator-independence (Neiland & Wilcock 1998). Though experimental studies of floral and pollination biology are required to confirm details of the breeding system in *N. nipponica*, it may not be necessary to make provision for management of insect service as part of a conservation strategy for the species.

The relative importance of regeneration from seed in the species' overall reproductive strategy also remains unclear. Despite the fact that approximately two-thirds of both populations remained sterile, seed production per flowering plant is high and could contribute to a significant seed bank. Presently, there is no data available for either *in* or *ex situ* germination rates of seeds of *Nervilia nipponica*. Nonetheless, the rarity of *N. nipponica* presents something of a paradox given the apparent efficiency of both its sexual and vegetative reproductive systems.

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